Oncogene (2004) 23, 2442–2453

www.nature.com/onc

# Transcriptional regulation by the estrogen receptor of antioxidative stress enzymes and its functional implications

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**We previously reported that antiestrogen-liganded estrogen receptor β (ERβ) transcriptionally activates the major detoxifying enzyme quinone reductase (QR) (NAD(P)H:-quinone oxidoreductase). Our studies also indicate that upregulation of QR, either by overexpression or induction by tamoxifen, can protect breast cells against oxidative DNA damage caused by estrogen metabolites. We now report on the upregulation of glutathione *S*-transferases Pi (GST-Pi) and gamma-glutamylcysteine synthetase heavy subunit (GCSh) expression by antiestrogens. Studies indicate the regulation of GST-Pi and GCSh transcriptional activity by ER. While ER regulation is mediated by an electrophile response element (EpRE), we identified mechanistic differences in the involvement of other transcription factors. Regardless of these differences, ERβ-mediated regulation of GST-Pi and GCSh point towards an important role for ERβ in cellular protection against oxidative stress. A protective role is supported by our observation of inhibition of estrogen-induced DNA damage upon upregulation of GST-Pi and GCSh expression.**
*Oncogene* (2004) **23,** 2442–2453. doi:10.1038/sj.onc.1207358
Published online 15 December 2003

**Keywords:** estrogen receptor; antiestrogens; glutathione-S-transferase; gamma-glutamylcysteine synthetase

## Introduction

Phase 2 detoxification enzymes such as NAD(P)H:(quinone-acceptor) oxidoreductase (quinone reductase (QR)), glutathione *S*-transferases (GSTs), epoxide hydrolase, and UDP-glucuronosyltransferases are induced in cells by electrophilic compounds and phenolic antioxidants (reviewed in Talalay, 1989; Prestera *et al*., 1993). These widely distributed enzymes detoxify electrophiles, thereby protecting cells against the toxic and neoplastic effects of carcinogens. We have previously shown that increases in QR enzyme activity can be induced by low concentrations of antiestrogens in breast cancer cells (Montano and Katzenellenbogen, 1997). Induction of QR enzymatic activity showed unusual reversed pharmacology, being markedly upregulated by antiestrogen and suppressed by estrogen in breast cancer cells. The antiestrogen regulation of QR enzymatic activity represents a potentially important pharmacological mechanism for this group of anticancer drugs that had not been previously recognized.

The electrophile response element (EpRE, also referred to as antioxidant response element, ARE) motif has been identified in the regulatory region of the gene encoding QR (reviewed in Jaiswal, 2000). This element has been shown to mediate basal expression and its activation by phenolic antioxidants, and it appears to be essential for antiestrogen stimulation (Montano and Katzenellenbogen, 1997). Since the EpRE is involved in the regulation of other genes encoding proteins involved in the protection against oxidative stress, we examined if these genes can also be regulated by estrogen receptor (ER) and its ligands. For these studies, we chose two genes in particular that encode antioxidative stress enzymes, glutathione *S*-transferase Pi (GST-Pi) and gamma-glutamylcysteine synthetase heavy subunit (GCSh). The functions of which are interrelated as will be discussed below.

The nonprotein thiol, glutathione (gamma-glutamylcysteinyl-glycine, GSH) is a predominant cellular antioxidant (reviewed in Griffith and Mulcahy, 1999). As such GSH serves critical functions in the maintenance of cellular redox balance and provides protection against reactive oxygen species. GSH is involved in the detoxication of xenobiotics either through direct reactions with reactive intermediates or via enzymatic conjugation reactions catalysed by GSTs. Exposure of cells to a number of xenobiotic agents results in a significant increase in the total intracellular GSH content. This is due to transcriptional upregulation of the genes encoding the two protein subunits (catalytic (heavy) and regulatory (light)) of gamma-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in its *de novo* synthesis. It is hypothesized that transcriptional upregulation of the two GCS subunit genes involves

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This work was supported by National Institute of Health Grant CA80959 to MMM
Received 28 April 2003; revised 13 October 2003; accepted 14 November 2003

similar *cis*-elements, but distinct combinations of *trans*-acting factors, contributing to differential regulation in response to specific inducting agents (Mulcahy *et al*., 1997; Moinova and Mulcahy, 1998). Transcription is hypothesized to involve dimeric transcription factors composed of small Maf proteins and various other bZIP family members, including NFE-2-related factor 1 and 2 (NRF1 and NRF2) (Wild *et al*., 1999).

GST is a family of enzymes that catalyses the conjugation of electrophilic compounds with glutathione (reviewed in Strange *et al*., 2001). This resulting complex is less toxic and more readily excreted. The GST gene family is extensive, with distinct isozymes expressing different functional properties. The family is characterized by promiscuous substrate specificity with low 'catalytic efficiency', characteristics integral to the evolution of GSTs as detoxifiers of a broad spectrum of endogenous and environmental chemicals. GST-P1-1, the only representative of the GST-Pi family of enzymes, is the most prevalent isozyme in nonhepatic tissues. The transcription factors Fos and Jun have been proposed to functionally interact and bind to promoter elements at the GST-Pi gene promoter (Moffat *et al*., 1994, 1996).

Antiestrogen upregulation of QR expression is reflected at the transcriptional level and requires the ER. Interestingly estrogen receptor $\beta$ (ER$\beta$) is a more potent activator of QR gene transcriptional activity than estrogen receptor $\beta$ (ER$\alpha$) (Montano *et al*., 1998). Gel shift assays suggest that antiestrogen-mediated induction of QR gene transcriptional activity in MCF7 cells involves a direct transcriptional effect where ER$\alpha$ or ER$\beta$ are components of the protein complex that binds the EpRE. Our studies indicate that the regulation by antiestrogen-liganded ER may also be attributable to changes in the levels and/or the activity of other factors (Montano *et al*., 2000) Supporting the involvement of other protein factors is that the same receptor isoform bound to the same ligand can have different transcriptional activities dependent on the enhancer element (ERE *vs* EpRE, Montano *et al*., 1998). Unraveling these additional EpRE binding proteins and how they affect ER binding and transcriptional activity are crucial for understanding how antiestrogen-bound ER causes upregulation of these genes. Thus, studies were conducted to further dissect the molecular mechanism(s) involved in antiestrogen induction of the GST-Pi and GCSh EpRE by identifying other transcriptional factors involved in this regulation. In addition, a biological readout for transcriptional regulation of GST-Pi and GCSh by antiestrogens was established by examining its role in the inhibition of estrogen-induced DNA damage.

We now report on the upregulation of GST-Pi and GCSh expression by antiestrogens. Studies indicate the regulation of GST-Pi and GCSh transcriptional activity by ER$\beta$. While ER$\beta$ regulation is mediated by an EpRE element, we identified mechanistic differences in the involvement of other transcription factors. Regardless of these differences, functional studies on ER$\beta$-mediated regulation of GST-Pi and GCSh point towards an important role for ER$\beta$ in cellular protection against oxidative stress.

## Results

### *Upregulation of GST-Pi and GCSh expression and transcriptional activity by estradiol and* trans*-hydroxytamoxifen (TOT)*

Using Northern blot analyses, we observed an average 1.9-fold increase in GST-Pi mRNA expression after tamoxifen treatment. (Figure 1a). The increase in GST-Pi expression in response to tamoxifen is also evident at the protein level wherein we observe an average ninefold increase (Figure 1b). GCSh mRNA expression was upregulated in MCF7 cells after estrogen (average 2.5-fold) and tamoxifen (average threefold) treatment (Figure 1a). GCSh protein expression was also upregulated by estrogens (average 2.6-fold) and antiestrogens (average 5.4-fold) (Figure 1b).

To determine if estrogen and antiestrogen regulation of GST-Pi expression occurs at the transcriptional level, a luciferase reporter construct containing $-242/+85$ GST-Pi gene promoter region was transfected into breast epithelial MDA-MB-231 cells along with an expression vector for ER$\alpha$ or ER$\beta$. It has been previously reported that the proximal promoter at $-130/-41$ has the elements necessary for optimal expression (Jhaveri and Morrow, 1998a, b). This region also contains a putative EpRE element. Our analyses of reporter activity revealed an increase in GST-Pi gene transcriptional activity in response to TOT (Figure 2a), with stronger activation observed with ER$\beta$ than with ER$\alpha$. While we observe an increase in reporter activity in

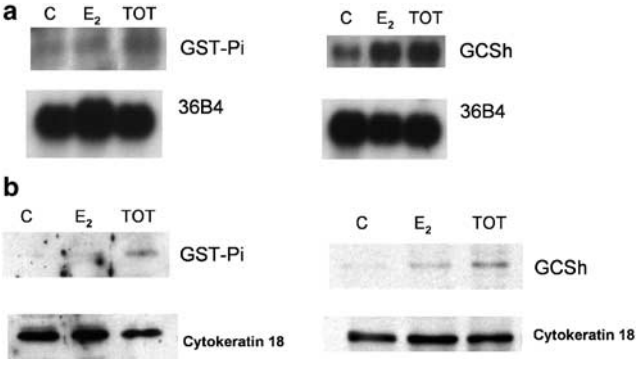


**Figure 1** Increased expression of GST-Pi and GCSh in response to estradiol and *trans*-hydroxytamoxifen. (**a**) Northern blot analyses of mRNA from MCF7 cells treated with control ethanol vehicle (c), 17$\beta$-estradiol ($E_2$, $10^{-8}$ M), or *trans*-hydroxytamoxifen (TOT, $10^{-7}$ M) for 24 h. The autoradiographs are representative of three separate experiments. (**b**) Western blot analyses of GST-Pi and GCSh protein levels in MCF7 cells in the presence of vehicle (c), $E_2$ ($10^{-8}$ M), or TOT ($10^{-7}$ M). Whole cell lysates were collected 24 h after treatment, electrophoresed on SDS–PAGE gels, transferred to nitrocellulose filters, probed with GST-Pi or GCSh antibody, and visualized using horseradish peroxidase-conjugated secondary antibody. The lower panels show the blots probed with cytokeratin 18 to show equal loading. The autoradiographs are representative of three separate experiments

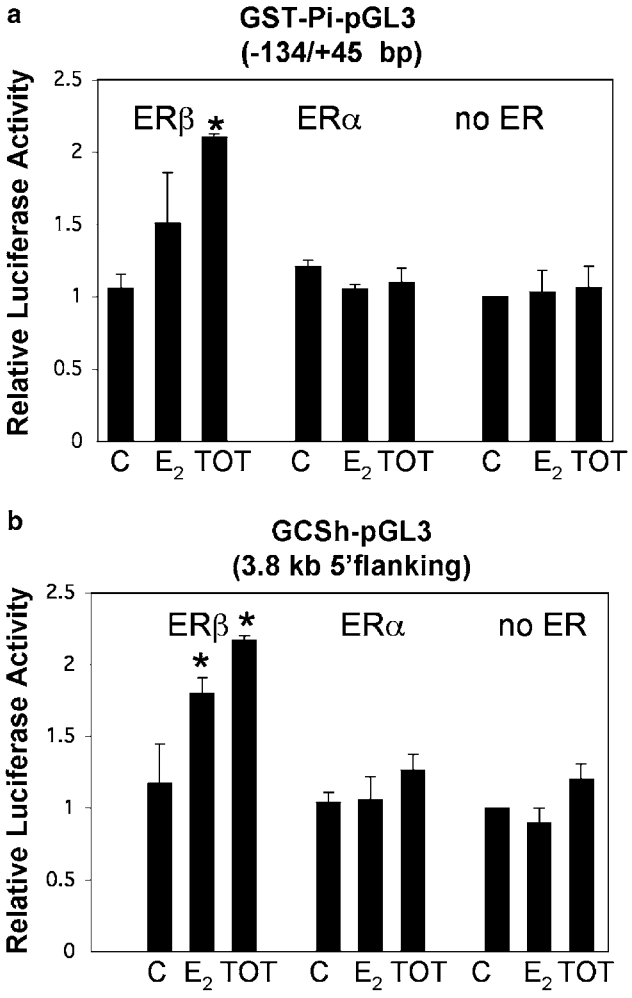


**Figure 2** Regulation of GST-Pi and GCSh gene promoter activity by the ER. MDA-MB-231 cells were transfected with the (**a**) GST-Pi gene or (**b**) GCSh gene promoter reporter constructs along with control expression vector (no ER, 100 ng) or expression vector for ER$\beta$ (100 ng), or ER$\beta$ (100 ng). Cells were also transfected with PRL-SV40/Luc internal control reporter to correct for transfection efficiency. Cells were then treated for 24 h with vehicle (c), E2 ($10^{-8}$ M), or TOT ($10^{-7}$ M) as indicated. Cell extracts were prepared and analysed for luciferase activity using firefly and *Renilla* luciferin substrate. The ratio of firefly to *Renilla* luciferase activity was calculated, and reporter activity in cells transfected with control expression vector (no ER) is set at 1. Values are the means $\pm$ s.e. from three or more separate experiments. * denotes level of significance $P<0.05$ versus control as determined by *t*-test

response to 17$\beta$-estradiol ($E_2$), the increase was not statistically significant.

A luciferase reporter construct containing the $-3.8$ kb 5′flanking region of the GCSh gene promoter was introduced into MDA-MB-231 cells. An increase in transcriptional activity of the GCSh-pGL3 reporter construct in the presence of $E_2$ and TOT was observed in MDA-MB-231 cells when cells were cotransfected with an expression vector for ER$\beta$ (Figure 2b). The induction of GST-Pi-pGL3 and GCSh-pGL3 activities appears to be mediated by the ER because no induction was observed in cells transfected with an empty expression vector. No increase in the activity of the control pGL3 promoter vector was observed with $E_2$ or TOT (data not shown).

*Identification of GST-Pi and ERβ GCSh gene promoter regions and transcription factors involved in the activation by the ER*

An EpRE core sequence G/CTGA(C/G)nnnGC(A/G) can be localized at $-70$ to $-60$ bp of the GST-Pi gene promoter. This putative EpRE also contains a putative AP1 site. In addition, an NF-$\kappa$B site, distal GC box, and proximal GC box have been identified at the $-125/-33$ bp region of the GST-Pi gene promoter (Jhaveri and Morrow, 1998a, b). To ascertain the involvement of the putative EpRE in ER$\beta$-mediated activation of the GST-Pi gene promoter, we performed deletional and mutational analyses of the $-125/-33$ bp region of the GST-Pi gene promoter. Removal of the NF-$\kappa$B site (at $-98/-89$) decreased basal reporter activity, but ER$\beta$ activation was still evident (Figure 3a). Mutations of the EpRE/TRE, distal GC (Sp1 element), and proximal GC box (Sp1 element) significantly decreased promoter activity; however TOT induction of GST-Pi gene promoter activity was still evident after mutations of the distal and proximal GC box (Figure 3b). No induction with TOT was apparent after mutation of the putative EpRE/AP1 site. A luciferase reporter vector wherein the EpRE was introduced upstream of a heterologous thymidine kinase (tk) gene promoter was also activated by TOT (Figure 3b). Upon mutation of the proximal GC box, we observed a more significant activation by $E_2$, suggesting that this element may have a repressive effect on $E_2$ activation. While the mechanism for the activation by estrogens is beyond the scope of this manuscript, Sp1 transcription factors have been shown to have both positive and inhibitory crosstalk with nuclear receptor-mediated transcription (reviewed in Safe, 2001; Zhang and Dufau, 2003).

An EpRE core sequence has been previously localized to $-3.1$ kb from the transcriptional start site of the GCSh gene promoter (Mulcahy *et al*., 1997). This EpRE was introduced upstream of the tk gene promoter cloned into the pGL3 vector to make $EpRE_{GCSh}$-tk-pGL3. After cotransfection of reporter into MDA-MB-231 cells, increased reporter activity was observed with both estrogen and tamoxifen (Figure 3c). Upon mutation of the EpRE core sequence, no induction was evident with either estrogen or tamoxifen (Figure 3c).

We then examined the effects of other transcription factors on EpRE enhancer activity. The two EpREs behaved similarly to the EpRE identified in the QR gene promoter as activation was observed with other factors previously identified to activate QR EpRE enhancer activity, NRF2 and hPMC2 (Figures 4a and b). The level of induction observed with ER$\beta$ was comparable to NRF2, which has been previously shown to upregulate GCSh EpRE enhancer activity. AP-1 transcription factors have been previously reported to bind to the GST-Pi EpRE (Moffat *et al*., 1994, 1996). However, this does not appear to result in transactivation as Jun, Fos, or Jun and Fos together did not activate GST-Pi or GCSh EpRE activity (Figure 4). As a control, Fos and Jun are shown in Figure 4c as capable of activating a luciferase reporter containing AP1 consensus binding sites.

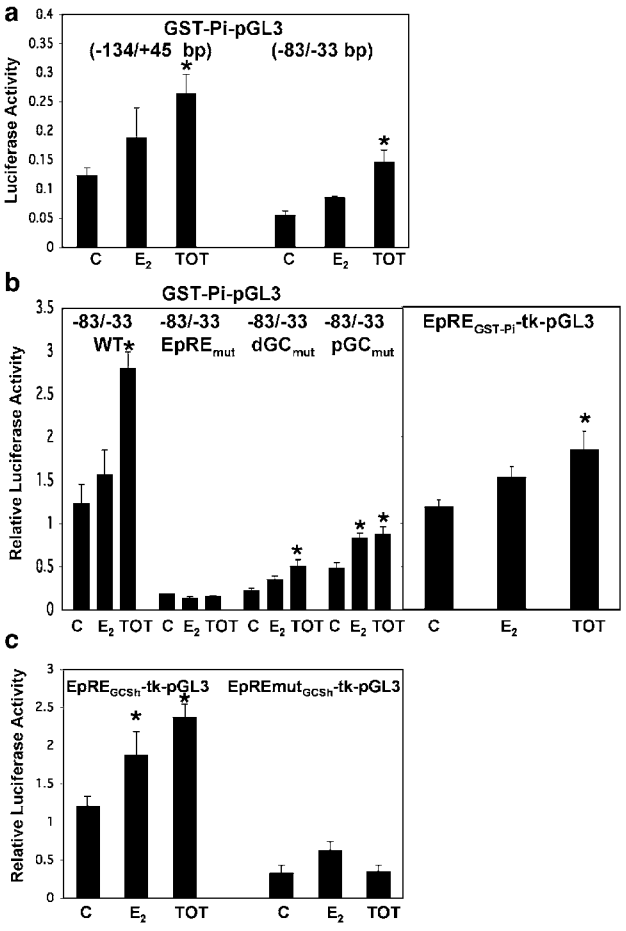


**Figure 3** ER-mediated regulation of GST-Pi and GCSh gene promoter activity can be localized to the EpRE. (**a**) −134/+45 or −83/−33-GST-Pi gene promoter reporter constructs were transfected into MDA-MB-231 cells along with an expression vector for ER$\beta$ (100 ng). * denotes level of significance $P<0.05$ versus control as determined by *t*-test. (**b**) Wild-type or mutant −83/−33-GST-Pi, or reporter construct containing the putative EpRE upstream of the tk gene promoter were transfected into MDA-MB-231 cells along with an expression vector for ER$\beta$ (100 ng). * denotes level of significance $P<0.05$ versus control as determined by *t*-test. (**c**) Reporter constructs containing wild-type or mutant GCSh EpRE upstream of the tk gene promoter were transfected into MDA-MB-231 cells along with an expression vector for ER$\beta$ (100 ng). * denotes level of significance $P<0.01$ versus control as determined by *t*-test. In (**a**)–(**c**), cells were treated for 24 h with control ethanol vehicle (c), $E_2$ ($10^{-8}$ M), or TOT ($10^{-7}$ M) as indicated. Cell extracts were prepared and analysed for luciferase activity using firefly and *Renilla* luciferin substrate. The ratio of firefly to *Renilla* luciferase activity was calculated. For each wild-type and mutant reporter construct, reporter activity is reported as fold induction over reporter activity in cells transfected with wild-type reporter and control expression vector (no ER) is set at 1. Each value represents the mean of three or more separate determinations ± s.e.m.

The involvement of NRF2 and AP1 in ER$\beta$-mediated regulation of EpRE activity was examined using dominant negative mutant NRF2 or Fos, respectively. We utilized amounts of $Fos_{DNM}$ and $NRF2_{DNM}$ expression vector that did not influence basal reporter activity. The amount of $Fos_{DNM}$ used was able to inhibit Fos activation of AP1 reporter (data not shown). As shown in Figure 5, both $NRF2_{DNM}$ and $Fos_{DNM}$

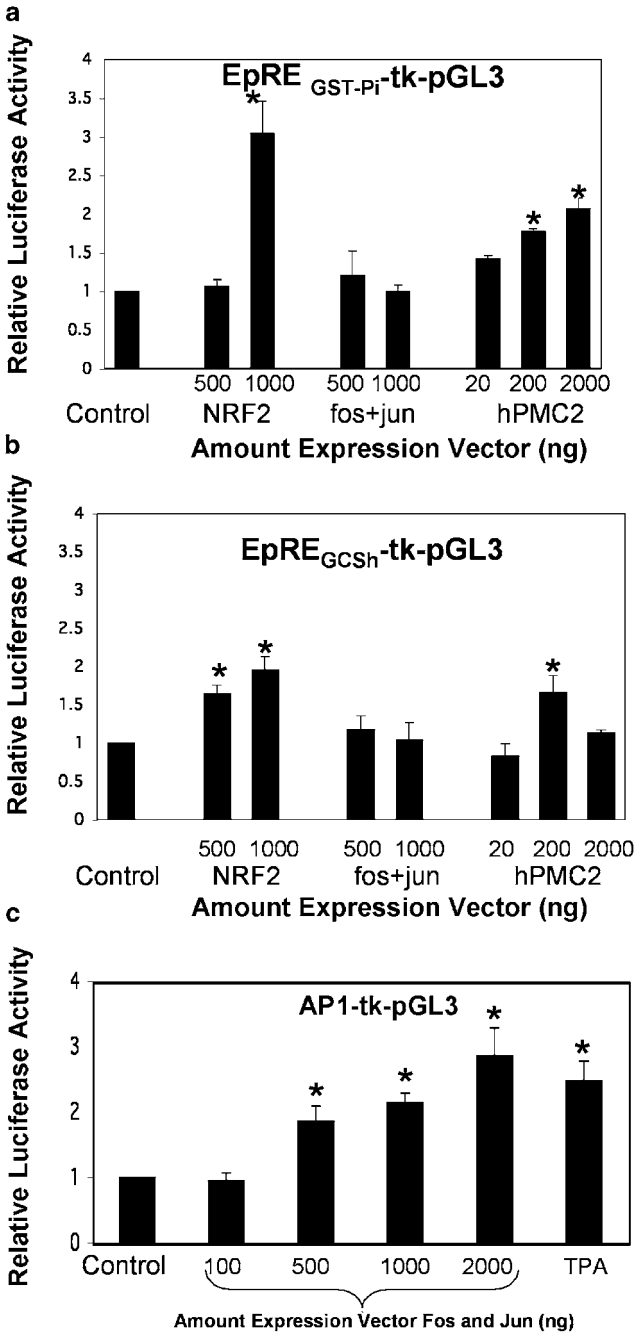


**Figure 4** Regulation of GST-Pi and GCSh EpRE activity by other activators of EpRE activity. MDA-MB-231 cells were transfected with (**a**) $EpRE_{GST\text{-}Pi}$-tk-pGL3 or (**b**) $EpRE_{GCSh}$-tk-pGL3 along with expression vectors for NRF2, Fos, and Jun, or hPMC2. In (**c**), MDA-MB-231 cells were transfected with AP1-luciferase reporter constructs along with indicated amounts each of Fos and Jun expression vectors. As a control, cells were treated with $10^{-7}$ M of the phorbol ester TPA. In (**a**)–(**c**), cells were also transfected with PRL-SV40/Luc internal control reporter to correct for transfection efficiency. Cell extracts were prepared and analysed for luciferase activity using firefly and *Renilla* luciferin substrate. The ratio of firefly to *Renilla* luciferase activity was calculated, and reporter activity in cells transfected with control expression vector is set at 1. Values are the means ± s.e. from three or more separate experiments. * denotes level of significance $P<0.01$ versus control as determined by *t*-test

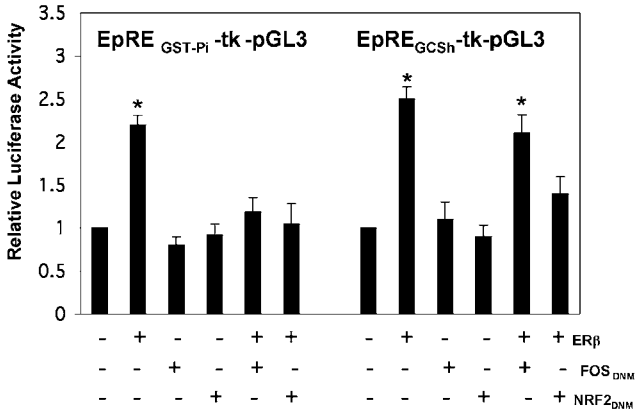


**Figure 5** Role of NRF2 and Fos transcription factors in ERβ-mediated activation of EpRE enhancer activity. MDA-MB-231 cells were transfected with $EpRE_{GST\text{-}Pi}$-tk-pGL3 or $EpRE_{GCSh}$-tk-pGL3 along with expression vectors for ERβ and/or dominant negative mutant NRF2 ($NRF2_{DNM}$, 500 ng) or dominant negative mutant Fos ($Fos_{DNM}$, 500 ng). Cells transfected with ERβ expression vector were also treated with TOT ($10^{-7}$ M) for 24 h. Cells were also transfected with PRL-SV40/Luc internal control reporter to correct for transfection efficiency. Cell extracts were prepared and analysed for luciferase activity using firefly and *Renilla* luciferin substrate. The ratio of firefly to *Renilla* luciferase activity was calculated, and reporter activity in cells transfected with control expression vector is set at 1. Values are the means ± s.e. from three or more separate experiments. * denotes level of significance $P<0.01$ versus control as determined by *t*-test

inhibited ERβ-mediated activation of GST-Pi EpRE activity, while only $NRF2_{DNM}$ inhibited activation of GCSh EpRE activity. Our results indicate that while Fos appears to be involved in ERβ regulation of GST-Pi EpRE, this does not appear to be the case for the GCSh EpRE (Figure 5). However, NRF2 appears to be involved in activation of both the GST-Pi and GCSh EpRE. These results suggest differential requirement for other transcription factors, dependent upon the EpRE and/or its flanking sequences, for ERβ activation.

### *Biochemical analyses of ERβ binding to GST-Pi and GCSh EPRE*

Gel shift assays were then conducted to determine if ERβ is part of the DNA–protein complex(es) that bind to the EpRE site. $^{32}$P-end-labeled GST-Pi EpRE or GCSh EpRE were incubated with MCF7 nuclear extracts or *in vitro* translated Fos, Jun, Nrf2, and/or purified recombinant ERβ. A representative autoradiograph in Figure 6a shows a GST-Pi EpRE–protein complex that can be competed out by EpRE and AP1 oligonucleotides and ERβ and NRF2 antibodies, but not a nonspecific IgG. This suggests that ERβ, AP1, and NRF2 transcription factors form a protein complex on the GST-Pi EpRE. Further studies indicate that ERβ alone did not bind to the EpRE (Figure 6b). While no band shift was observed with either *in vitro* translated Fos or Jun (data not shown), a DNA–protein complex (SB) was observed when both Fos and Jun were added to the reaction. The intensity of the band was stronger in the presence of ERβ. The specificity of the DNA–protein interactions was verified using competitive gel shift assays with unlabeled GST-Pi EpRE. We also observed competition by unlabeled AP1 and ERE, suggesting the presence of Fos, Jun, and ER in the DNA–protein complex. In addition, the DNA–protein complex was competed out by ERβ antibody, suggesting that ERβ interacts with Fos and/or Jun on the GST-Pi EpRE. Thus, while Fos and Jun do not directly activate GST-Pi EpRE, it may be involved in the recruitment of other factors, such as ERβ, that can activate the EpRE. A DNA–protein complex was also observed in the presence of ERβ and NRF2 that can be competed out with either ERβ or NRF2 antibodies. This observation suggests interactions between ERβ and NRF2 on the GST-Pi EpRE as well. No binding by Fos and Jun, or ERβ with Fos and Jun were evident upon mutation of the EpRE (data not shown).

Gel shift assays using MCF7 nuclear extracts along with the GCSh EpRE also suggest the presence of AP1, ERβ, and NRF2 in a protein complex bound to the EpRE (Figure 6c). However, our studies also suggest that Fos and Jun do not interact with ERβ on the EpRE (Figure 6d). No appreciative binding of ERβ was evident with GCSh EpRE, but a DNA–protein complex was observed in the presence of ERβ and NRF2 that can be competed out with either ERβ or NRF2 antibodies. Thus NRF2, but not Fos and Jun, may interact with ERβ on the GCSh EpRE. Neither NRF2 nor hPMC2 interact with Fos and Jun on the GCSh or GST-Pi EpRE. This is based on gel shift assays wherein the AP1–DNA complex (complex A, Figure 6b and d) was not competed out by NRF2 antibody, and the NRF2–EpRE complex (complex B, Figure 6b and d) was not competed out by AP1 oligonucleotides.

### *Role of GST-Pi and GCSh in the protection against estrogen-induced DNA damage*

We have previously reported that physiological concentrations of $E_2$ cause oxidative DNA damage (as measured by levels of 8-hydroxydeoxyguanine, 8-OH-dG) in breast epithelial cells, which is dependent upon estrogen metabolism (Bianco *et al*., 2003). TOT and the pure antiestrogen ICI-182,780 protected against $E_2$-mediated damage in MCF7 cells containing ERβ. This is most likely due to the ability of these antiestrogens to activate expression of QR via ERβ. Consistent with this is our observation that levels of 8-OH-dG were inversely correlated to QR and ERβ levels. However, our studies indicate that QR may not be the only antioxidative stress enzyme involved in this protective effect. We thus examined if GST-Pi or GCSh can protect against $E_2$-induced DNA damage.

We used retroviral infection to modulate GST-Pi and GCSh levels in MCF7 cells, and changes in protein levels were demonstrated using immunofluorescence staining (Figure 7a). Consistent with our previous studies, we see upregulation of 8-OH-dG with $E_2$ treatment, and inhibition of $E_2$-induced DNA damage by TOT (Figure 7b). Upregulation of GST-Pi (average 75% increase) or GCSh (average 83% increase) levels

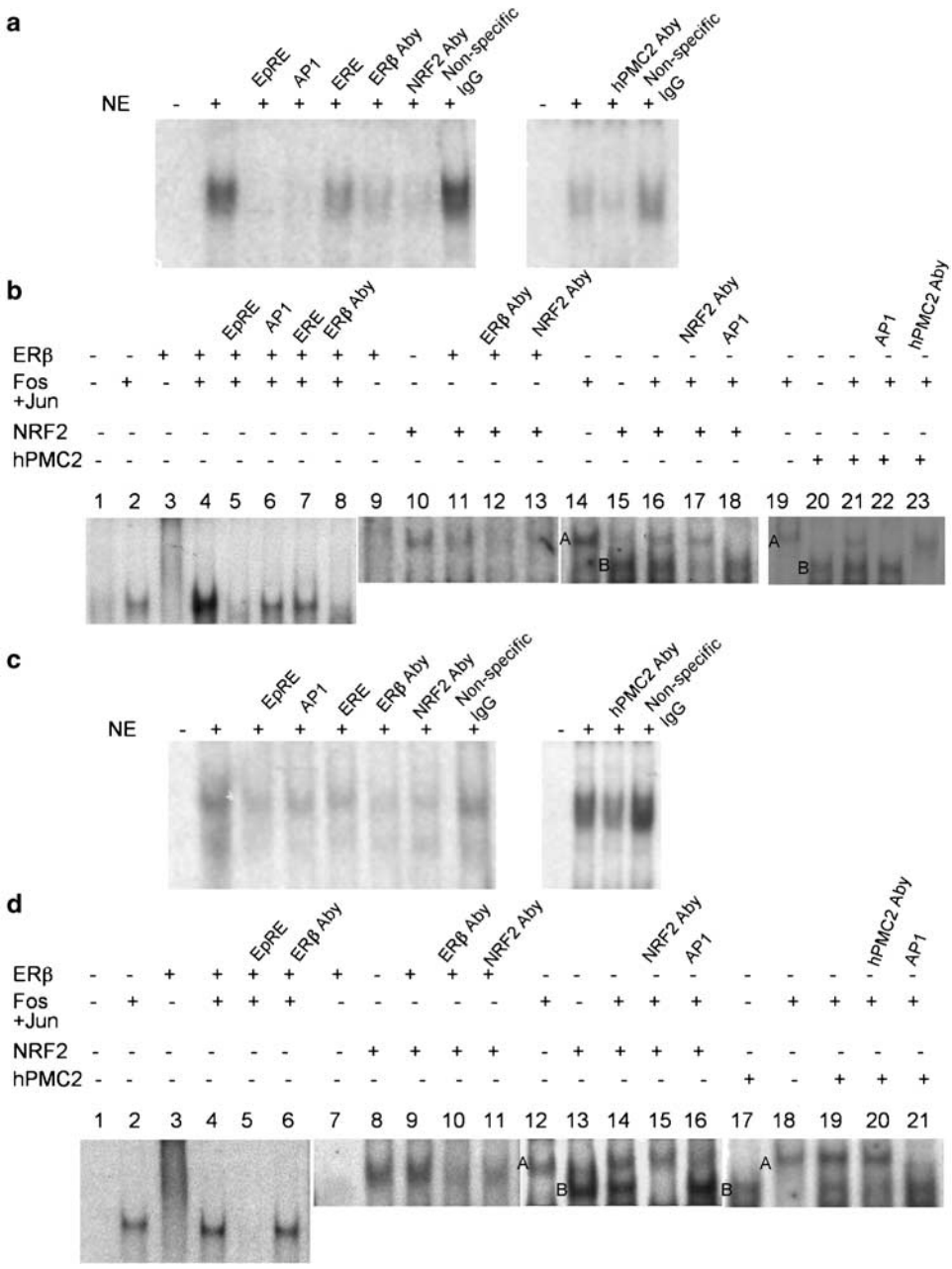


**Figure 6** Fos, Jun, and ER$\beta$ form a complex on the GST-Pi EpRE but not on the GCSh EpRE. Gel mobility shift assays were performed using a double-stranded oligomer containing the EpRE of the human GST-Pi gene or the human GCSh gene. $^{32}$P-EpRE GST-Pi were incubated with (**a**) MCF7 nuclear extracts or (**b**) *in vitro* translated Fos, Jun, NRF2, hPMC2, and/or purified recombinant ER$\beta$ in the absence or presence of 100-fold excess of unlabeled EpRE, AP1, ERE, monoclonal NRF2 antibody, monoclonal ER$\beta$ antibody, polyclonal hPMC2 antibody, or nonspecific IgG. $^{32}$P-EpRE GSCh were incubated with (**c**) MCF7 nuclear extracts or (**d**) *in vitro* translated Fos, Jun, NRF2, hPMC2, and/or purified recombinant ER$\beta$ in the absence or presence of unlabeled EpRE, AP1, ERE, monoclonal NRF2 antibody, monoclonal ER$\beta$ antibody, polyclonal hPMC2 antibody, or nonspecific IgG. Equal c.p.m. and ng amounts of $^{32}$P-EpRE GST-Pi or $^{32}$P-EpRE GCSh were used in the binding reactions. The autoradiographs are representative of three separate experiments

while not affecting basal 8-OH-dG levels led to inhibition of $E_2$-induced DNA damage. These results suggest a similar protective role as QR. While slightly higher 8-OH-dG levels were observed in GST-Pi- or GCSh-infected cells treated with $E_2$ and TOT when compared to cells treated with $E_2$ alone, the increases were not significant.

To determine the role of GST-Pi and GCSh in tamoxifen protection against DNA damage, we down-regulated GST-Pi (average 43% decrease) or GCSh (average 46% decrease) expression using GST-Pi antisense (GST-Pi$_{AS}$) or GCSh antisense (GCSh$_{AS}$) retroviruses, respectively. Decreases in GST-Pi or GCSh expression led to a significant increase in basal 8-OH-dG

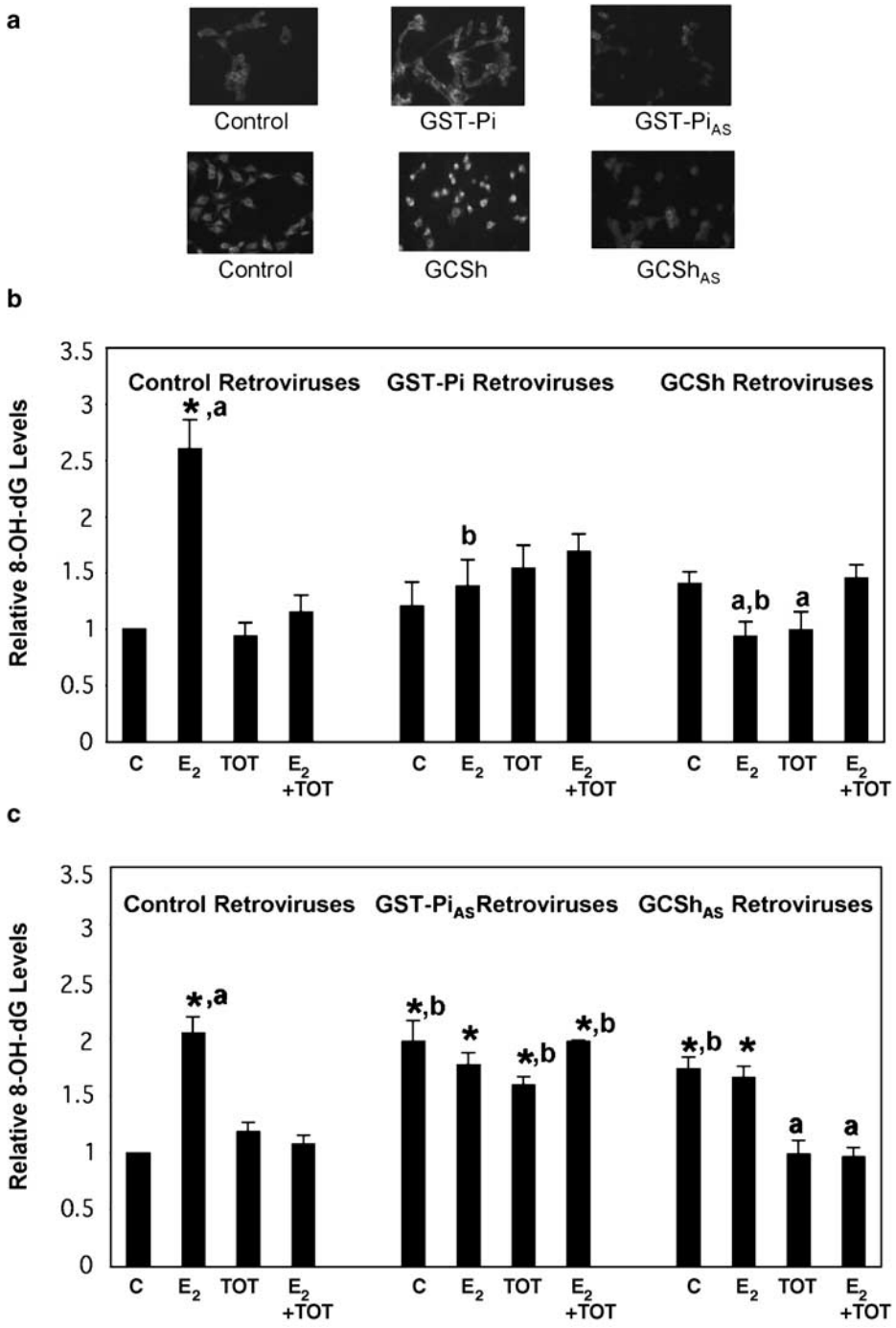


**Figure 7** GST-Pi and GCSh protect against estrogen-induced oxidative DNA damage. (**a**) GST-Pi or GST-Pi$_{AS}$ retrovirus-infected MCF7 cells were immunostained for GST-Pi. GCSh or GCSh$_{AS}$ retrovirus-infected MCF7 cells were immunostained for GCSh. Control retroviruses were obtained from cells transfected with the pBPSTR1 vector alone. (**b**) MCF7 cells were transiently infected with GST-Pi, GCSh, or control retroviruses. (**c**) MCF7 cells were transiently infected with GST-Pi$_{AS}$, GCSh$_{AS}$, or control retroviruses. The cells in (**b**) and (**c**) were then treated with vehicle (control), $E_2$ ($10^{-8}$ M), and/or the antiestrogen TOT($10^{-7}$ M) for 24 h and immunostained for 8-OHdG. * denotes level of significance $P<0.01$ versus vehicle-treated control cells as determined by *t*-test. a denotes level of significance $P<0.05$ versus respective control as determined by t-test. b denotes level of significance $P<0.01$ versus cells infected with control retroviruses with same treatment as determined by *t*-test.

levels (Figure 7c). No further increase in 8-OH-dG levels was observed in these cells after $E_2$ treatment. It is possible that by decreasing GST-Pi or GCSh expression, optimal induction of 8-OH-dG levels has already been achieved. TOT was not able to protect against estrogen-induced DNA damage after inhibiting GST-Pi expression. However, GCSh$_{AS}$-infected cells treated with $E_2$ and tamoxifen had 8-OH-dG levels that were

significantly lower compared to what was observed with estrogen alone. These findings suggest that while overexpression of GST-Pi or GCSh can protect against DNA damage, tamoxifen-mediated protection can be attributed more to induction of GST-Pi than GCSh. It is possible that cells are more able to compensate for the decrease in GCSh expression. GST-Pi and QR may also be more directly involved in inactivating estrogen metabolites, thus decreases in their expression may have more significant effect on 8-OH-dG levels. Of note, the upregulation of GCSh gene transcription by estrogen were not able to counteract estrogen-induced DNA damage.

## Discussion

We have previously performed extensive studies on the regulation of QR transcriptional activity by ER and its functional implications. We now report that ER$\beta$ also regulates GST-Pi and GCSh gene transcriptional activity through the EpRE element in their promoter region. These findings further validate our findings of ER regulation of EpRE enhancer activity. However, our findings also indicate mechanistic differences in the regulation of GST-Pi EpRE and GCSh EpRE by the ER$\beta$. While NRF2 appears to interact with ER$\beta$ on GST-Pi and GCSh EpRE, our studies support the differential involvement of AP1 transcription factors in ER$\beta$ binding and activation of promoter activity through the EpRE of different genes. It is possible that the recruitment of ER$\beta$ to the EpRE of different gene promoters may be dependent on different factors. Further functional studies support a protective role for GST-Pi and GCSh against estrogen-induced oxidative DNA damage.

As a major cellular antioxidant, GSH maintains cellular redox balance and protects against reactive oxygen species. GSH formation is controlled by the actions of the enzymes GCS and glutathione synthetase, with the former enzyme catalysing the rate-limiting step (reviewed in Griffith and Mulcahy, 1999). GCS is a holoenzyme comprised of a heavy chain (GCSh, 73 kDa) and a light subunit (GCSl, 28 kDa). The light subunit functions to stabilize the enzyme and is therefore termed the regulatory chain. As the entire catalytic activity of the enzyme resides within the heavy chain, regulation of glutathione synthesis is closely correlated with GCSh gene expression. GST-Pi belongs to a family of enzymes that catalyse the conjugation of electrophilic compounds with glutathione (reviewed in Strange *et al*., 2001). The expression of antioxidative stress enzymes is regulated by common promoter elements, the focus of this manuscript being the EpRE/ARE. The three enzymes that we have examined, QR, GST-Pi, and GCSh, appear to be regulated at the transcriptional level by the ER as well.

The EpRE in the GST-Pi promoter functions similarly to EpREs identified in the QR in that activation was observed with NRF2 and hPMC2. The existence of a functional EpRE is supported by *in vivo* observations that GST-Pi expression can be induced in the livers of rats fed a diet consisting of the antioxidant butyrated hydroxyanisole (BHA) (Cha and Heine, 1982). Our studies are also consistent with a functional interaction between Fos and Jun and ER$\beta$ in the activation of EpRE enhancer activity. Consistent with previous reports is our observation of the inability of Fos and Jun, individually or together, to activate the putative AP1 sequence in GST-Pi gene promoter (Morrow *et al*., 1990). The functional interaction between AP1 transcription factors and ER has been well characterized and has been implicated in the differential activation of ER$\alpha$ and ER$\beta$ by estrogens and antiestrogens (Paech *et al*., 1997). In the context of a consensus AP1 site, ER$\beta$ is activated by tamoxifen. In the context of the QR EpRE, Jun heterodimerizes with NRF2 and activates the QR and GST-Ya EpRE, while Fos appears to negatively regulate the QR EpRE and GST-Ya EpRE enhancer activity (reviewed in Dhakshinamoorthy *et al*., 2000; Jaiswal, 2000). A repressive role for Fos is supported by the increased expression of QR and GST-Ya EpRE in Fos knockout mice (Wilkinson *et al*., 1998). Our studies suggest that in the context of the GST-Pi gene promoter, Fos and Jun do not activate the GST-Pi EpRE but appear to be involved in recruiting the ER$\beta$ to the EpRE as well as ER$\beta$-mediated transcriptional activation of EpRE.

The induction observed with antiestrogen-liganded ER$\beta$ is not due to removal of repressor/silencing activity associated with the $-105/-86$ region, which also contains the NF-$\kappa$B-like element. Protein factors that bind to the repressor element at $-105/-86$ of the GST-Pi gene have been proposed to functionally interact with activator elements, Fos and Jun, that bind to the EpRE/TRE (Moffat *et al*., 1994). Previous reports suggest suppression of GST-P1 gene by other nuclear receptors by competitive binding inhibition between Fos/Jun and RAR-RA (Xia *et al*., 1993). That basal reporter activity did not increase upon removal of the $-242/-83$ bp region can also be attributed to the artificial nature of our reporter constructs. Previous reports indicate that GST-Pi expression can be observed after stably integrating a GST-Pi minigene that contains the putative silencer element in MCF7 cells despite the lack of endogenous GST-Pi gene expression (Jhaveri and Morrow, 1998a, b). This observation supports the involvement of other alterations in DNA or chromatin, such as methylation. A cluster of methylation sites has been identified around the transcriptional start site, and more dense methylation was observed in ER-positive than ER-negative cells (Jhaveri and Morrow, 1998a, b). Thus, in cultured human breast cancer cell lines, GSTP1 is exclusively expressed in ER-negative cells but is undetectable in ER-positive cells. Epigenetic silencing through promoter hypermethylation of GST-Pi gene is associated with breast, prostate, and renal cancer (Esteller *et al*., 1998; Singal *et al*., 2001).

It is possible that the expression of GST-Pi is regulated by the balance of two factors—methylation

and regulation by *trans*-acting factors. It has been proposed that GST-Pi activation might involve prior reversal of hypermethylation before *trans*-acting factors can act on the promoter. Roles for both methylation and *trans*-acting factor have been shown in the transcriptional regulation of several genes. For example, the methylation status of the ER gene promoters correlated well with the suppression of the expression of this gene in human breast cancer tissue as well as in human breast cancer cell lines (Yoshida *et al*., 2000). Methylation of ER gene promoters directly affects ER activity in breast cancer cells, but other mechanisms, such as loss of critical transcriptional factors, may also be at work in some cases (Yoshida *et al*., 2000). The telomerase gene is another gene that is silenced by methylation in mammalian cells but its expression appears to be upregulated by the ER in ovarian, breast, and prostate cells (Kyo *et al*., 1999; Misiti *et al*., 2000; Nanni *et al*., 2002). The EpRE in GST-Pi promoter lacks CpG sequences, thus it is unlikely that CpG methylation has a direct effect on transcription factor binding. Moreover, methylation of binding sites does not appear to interfere with binding and activation by certain transcription factors (reviewed in Singal and Ginder, 1999).

The molecular mechanism of GCSh upregulation has been extensively studied. The transcriptional regulation of GCSh appears to be dependent on the stimulus and the cell type (Rahman and MacNee, 1999; Dahl and Mulcahy, 2001). Studies by Mulcahy *et al*. (1997) have suggested that basal GCSh expression in liver HepG2 cells is controlled by EpRE, which lies 3.1 kb upstream from the start site of the gene. Transcriptional activation of GCSh and GSCl gene promoters were reported to be mediated by NRF2. Moreover, NRF2 is also involved in ER$\beta$-mediated activation of GCSh EpRE. Our studies also indicate differences in mechanism of transactivation between the GST-Pi and GCSh EpREs in that AP-1 transcription factors are not involved in ER$\beta$-mediated activation of GCSh gene transcription. Support for the involvement of ER in the regulation of GCSh is a report that phytoestrogens increase intracellular total GSH level in vascular smooth muscle cells (Mizutani *et al*., 2000).

While upregulation of QR and GST-Pi expression and transcriptional activity is observed only with antiestrogen-liganded ER, results from the present studies indicate induction GCSH transcriptional activity by estrogen-liganded ER as well. This is reflected at the mRNA and protein level. These observations suggest that the interaction of the estrogen-liganded ER with promoter-specific transcription factors mediate transactivation from the GCSH gene promoter.

We have now observed antiestrogen-mediated activation of EpRE enhancer activity in various promoter contexts, the QR gene promoter (Montano *et al*., 1998), GST-Ya subunit gene promoter (Montano and Katzenellenbogen, 1997), GST-Pi gene promoter, and GCSh gene promoter. The induction of QR gene transcriptional activity by antiestrogens was also evident in more than one cell context. It is of note that tamoxifen has been reported to induce an increase in the mRNA levels of other phase II detoxification enzymes in rat liver (Hellriegel *et al*., 1996). Our findings suggest that antiestrogens by regulating the expression of numerous proteins that contain EpREs in their regulatory regions may afford substantial chemoprotective benefit to ER-containing cells. This was tested by examining if GST-Pi and GCSh can protect against estrogen-induced DNA damage, as we have already observed for QR. We observe that upregulation of GST-Pi and GCSh can inhibit estrogen-induced oxidative DNA damage. Furthermore GST-Pi, like QR, can mediate antiestrogen inhibition of estrogen-induced DNA damage. While we observe significant changes in oxidative DNA damage levels with modulation of either GST-Pi and GCSh expression, the change observed is not as significant as TOT alone. These findings suggest induction of more than one antioxidative stress enzymes is involved in the prevention of DNA damage by tamoxifen.

Based on our results, it is expected that inactivation of GST-pi and GCSh would make breast epithelial cells susceptible to estrogen-induced DNA damage by compromising the ability of these enzymes to neutralize the electrophilic intermediates generated from estrogen metabolism. In line with the protective role of GST-Pi, polymorphism in the GST-Pi locus has been associated with breast cancer risk (Maugard *et al*., 2001; Mitrunen *et al*., 2001). However, therapeutic approaches that stimulate expression of antioxidative stress enzymes may be a case of a 'two-edged' sword. Glutathione (GSH) and related enzymes also play roles in cellular resistance to chemotherapy (reviewed in Tew, 1994). Expression of GST-Pi is associated with resistance to some antineoplastic drugs. Increased metabolic synthesis of glutathione was shown in drug-resistant MCF7 (Gamcsik *et al*., 2002). The increased synthetic rates of GSH in resistant lines reflected, in part, contributions from increased activities of GCS. An association between certain GCSh alleles and/or drug sensitivity has also been reported (Walsh *et al*., 2001), providing evidence that suggests polymorphism of human GCSh is functionally significant.

The identification of factors that regulate EpRE enhancer activity is important not only for understanding the regulation QR activity in cancer cells but also several other genes involved in antioxidant defenses. The observation that antiestrogens can regulate the expression of several antioxidative stress enzymes has broad implications regarding the potential beneficial effects of antiestrogens. Antiestrogens, such as tamoxifen and raloxifene, are routinely used for the treatment of breast cancer based on their ability to bind to the ER and block the proliferative effects of estrogens on the breast. In 1998, tamoxifen became the first drug to be approved for the reduction of risk for breast cancer. Upregulation of antioxidative stress enzymes may provide novel insights as to how tamoxifen prevents breast cancer and is important for developing newer and safer drugs.

## Materials and methods

### *Chemicals and materials*

Cell culture media were purchased from GIBCO (Grand Island, NY, USA). Calf serum was from Hyclone Laboratories (Logan, UT, USA) and fetal calf serum from Atlanta Biologicals (Norcross, GA, USA). $E_2$, TOT, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma Chemical Company. Custom oligonucleotides were purchased from Genosys (Grand Island, NY, USA).

### *Plasmids*

The reporter construct containing GST-Pi gene promoter region $-242/+85$ has been previously described (Singal *et al*., 2001). The GST-Pi-1 and GST-Pi-2 gene promoter reporter constructs representing $-124/-84$ and $-83/-33$, respectively, were constructed using the following oligonucleotides: 5′-cgcgcagcggccgccggggctggggccggcgggagtccgcgggaccctccagaag-3′, 5′-cgcgagcggccggcgccgtgactcagcactggggcggagcggggcgggaccaccc-3′.

The oligonucleotides were annealed with their complementary oligonucleotide, gel purified and cloned into *Mlu*I/*Xho*I-digested pGL3 vector. Reporter constructs containing mutations within $-83/-33$ were constructed using the following oligonucleotides: 5′-cgcgagcggccggcgccgcagtctggcactggggcggagcggggcgggaccaccc-3′, 5′-cgcgagcggccggcgccgtgactcagcactaaaataaagcggggcgggaccaccc-3′,5′-cgcgagcggccggcgccgtgactcagcactggggcggagcaaaataagaccaccc-3′.

The oligonucleotides were annealed with their complementary oligonucleotide, gel purified and cloned into *Mlu*I/*Xho*I-digested pGL3 vector. To make the reporter construct containing the GST-Pi EpRE upstream of the heterologous thymidine kinase (tk) promoter, the oligonucleotide 5′-cgcgggcgccgtgactcagcactggg-3′ was annealed with its complementary oligonucleotide, gel purified and cloned into *Mlu*I/*Xho*I-digested tk-pGL3 vector. The tk-pGL3 vector was constructed by subcloning the tk promoter containing *BamH*I/*Bgl*II insert from pTZ-tk into *Bgl*II-digested pGL3.

Reporter construct containing the 5′ flanking sequence ($-3.8$ kb) of the GCSh gene has been described previously (Wild *et al*., 1999). Reporter constructs containing the GCSh EpRE (wild type or mutant) were constructed using the following oligonucleotides: Wild-type EpRE: 5′-ctccccgtgactcagcgctttg- 3′, Mutant EpRE: 5′-ctccccgggactcagcgctttg-3′.

The oligonucleotides were annealed with their complementary oligonucleotide, gel purified and cloned into *Mlu*I/*Xho*I-digested tk-pGL3 vector. The AP1-luciferase reporter was obtained from Dr Clark Distelhorst (CWRU).

Expression vectors for wild-type human ER$\alpha$, ER$\beta$, and hPMC2 have been described previously (Montano and Katzenellenbogen, 1997; Montano *et al*., 2000). The expression vectors for Fos, Jun, and AFos were obtained from Dr Clark Distelhorst (CWRU). The expression vectors for NRF2 and dominant negative NRF2 were obtained from Dr Jeffrey Chan (UCSF) and Dr Jawed Alam (Alton Ochsner Medical Foundation), respectively.

To make the retroviral vector pBPSTR1-GST-Pi (sense or antisense), GST-Pi cDNA was released from pCI-Neo-GST-Pi (kindly provided by Dr Rakesh Singal) by *Nhe*I/*Xba*I digestion, blunted, and inserted into *Bam*HI-digested and blunted pBPSTR1 vector (Paulus *et al*., 1996). To make pBPSTR1-GCSh (sense or antisense), cDNA containing GCSh open reading frame was obtained using Access RT–PCR kit from Promega (Madison, WI, USA) according to the manufacturer's recommendation and using the upstream (gcggccatggggctgctgtcccagggctcgccg) and downstream (gaatgtctagttggatgagtcagttttacttcc) primers. The cDNA was then cloned into the pCR-Blunt II-TOPO vector to make pCRII-TOPO-GCSh using the Zero Blunt TOPO PCR Cloning Kit from Invitrogen (Carlsbad, CA, USA) and sequenced using the Sequenase Kit (United States Biochemicals, Cleveland, OH, USA). To construct pBSTR1-GCSh (sense or antisense), pCRII-TOPO-GCSh was digested with *Eco*RI to release GCSh coding region. The fragment was then blunted and inserted into *Bam*HI-digested and blunted pBPSTR1 vector.

### *Tissue culture*

Breast epithelial cells (MCF7 and MDA-MB-231) and PA317 amphotropic packaging cells were obtained from ATCC (Manassas, VA, USA) and maintained according to their recommended protocols. Prior to the experiments, breast epithelial cells were depleted of estrogen by growth in Improved minimal essential media minus phenol red containing 5% CDCS for 5 days before experiments.

### *Northern blot analyses*

Total RNA was isolated using Trizol (GIBCO BRL, Rockville, MD, USA). Gel purified reamplified GST-Pi and GCSh cDNA were random primer labeled using the Ready-to-Go DNA labeling kit from Pharmacia (Piscataway, NJ, USA) for Northern analysis. mRNA was separated by electrophoresis, transferred to nitrocellulose support, and hybridized with random primer-labeled cDNA (Montano *et al*., 1998). Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

### *Western blot analyses*

Whole cell extracts were prepared from breast epithelial MCF7 cells using M-PER (Pierce, Rockford, IL) Total protein concentration was determined using the BCA protein assay kit. Proteins were separated by electrophoresis on SDS–polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes. Blots were incubated with anti-GST-Pi polyclonal antibody (1 : 2000 dilution; Stressgen Biotechnologies, Victoria, BC, Canada) or GCSh polyclonal antibody (1 : 5000 dilution, Liu *et al*., 1998) and goat anti-rabbit IgG secondary antibody (1 : 40 000 dilution for GST-Pi and 1 : 30 000 for GCSh) for detection by chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ, USA).

### *Transfections*

MDA-MB-231 were transfected as previously described (Montano and Katzenellenbogen, 1997). Cells were seeded for transfection in 60-mm dish in improved minimum essential media (IMEM) minus phenol red containing 5% CDCS. Cells at 30–50% of confluence were transfected by the CaPO4 coprecipitation method 48 h later with 2 $\mu$g reporter constructs and 100 ng ER$\alpha$ or ER$\beta$ expression vector. Transfection efficiency was monitored by cotransfection with 150 ng pRL-SV40/Luc internal control plasmid. PRL-SV40/Luc contains cDNA (Rluc) encoding *Renilla* luciferase regulated by the SV40 enhancer and early promoter elements. Carrier DNA pTZ19R was added to adjust total DNA to 8 $\mu$g. Cells remained in contact with the precipitate for 5 h and were then subjected to a 2.5-min glycerol shock (20% in IMEM minus phenol red plus 5% CDCS). Cells were rinsed with HBSS and given fresh media with or without hormones. All cells were harvested 24 h after hormone treatment. Cells were rinsed once

at room temperature with PBS, lysed in 100 $\mu$l 1 × lysis buffer (Dual-Luciferase Assay System, Promega Corp.), and stored at −70°C until assayed. Luciferase activity was measured using 100 $\mu$l each of firefly and *Renilla* luciferin substrate (Promega Corp.) per 20 $\mu$l cellular lysate in a luminometer. Data are expressed as the ratio of firefly to *Renilla* luciferase activity.

*Gel shift assays*

Cells used to make nuclear extracts were harvested in cold PBS. Cells were then washed in PBS twice, pelleted, and resuspended in cold Buffer A with protease inhibitors (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 M PMSF, 2 $\mu$g/ml Leupeptin, 0.03 TiU/ml Aprotonin, 1 $\mu$g/ml Pepstatin). The cells were allowed to swell on ice for 15 min, after which 62.5 $\mu$l of 10% NP-40 was added to cell suspension. The suspension was then vortexed vigorously, pelleted and resuspended in Buffer B (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 M EDTA, 1 mM DTT, 0.5 M PMSF, 2 $\mu$g/ml Leupeptin, 0.03 TiU/ml Aprotonin, 1 $\mu$g/ml Pepstatin). The suspension was incubated at 4°C for 1 h, pelleted, and the supernatant containing nuclear extracts were collected and stored at −80°C.

Single-stranded oligomers containing the GST-Pi EpRE 5′-gcgccgtgactcagcactggg-3′ or GCSh EpRE 5′-ctccccgtgactcagcgctttg-3′ were annealed to their complement. The resultant double-stranded oligomer was gel purified on a nondenaturing 4.5% polyacrylamide gel run in 0.5 × TBE. The ability of purified protein(s) to bind to the GST-Pi EpRE or GCSh EpRE was analysed using gel mobility shift assays as described previously (Montano *et al*., 1998). Briefly, 4 $\mu$g of nuclear extracts or 4 $\mu$l (10–100 ng) of recombinant or *in vitro* translated proteins were mixed with 1 ng of end-labeled GST-Pi EpRE or GCSh EpRE oligomers in the presence of 0.4 $\mu$g/$\mu$l dIdC, 20 mM HEPES, 200 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 2 mM EDTA, 20% glycerol, 1 $\mu$g/$\mu$l BSA and incubated at room temperature for 20 min. The specificity of binding was assessed by competition with excess unlabeled double-stranded GST-Pi EpRE or GCSh EpRE. Protein–DNA complexes were analysed using nondenaturing gels. The sequence of the ERE and AP1 oligonucleotides were reporter previously (Montano *et al*., 1998). ER$\beta$ and NRF2 antibodies and nonspecific IgG were obtained from Santa Cruz Biotechnology. hPMC2 antibody was produced for our laboratory by Biosynthesis (Lewisville, TX, USA) using a peptide representing amino acids 170–187 of hPMC2.

In vitro *transcription and translation*

*In vitro* transcription and translation of Fos, Jun, and NRF2 were performed using the Promega TNT kit (Madison, WI, USA). Briefly, 1 $\mu$g of cDNA was mixed with 25 $\mu$l TNT rabbit reticulocyte lysate, 2 $\mu$l TNT buffer, 1 $\mu$l of amino-acid mixture, and 1 $\mu$l T3 RNA polymerase (20 U/$\mu$l). The final reaction of 50 $\mu$l was incubated for 90 min at 30°C.

*Retroviral-mediated transfection*

Retroviruses were made by transfecting PA317 cells with the pBPSTR1 plasmid alone or pBPSTR1 containing full-length GST-Pi or GCSh cDNAs (in the sense or antisense orientation). Breast epithelial cell lines were infected with retrovirus-containing supernatants in the presence or absence of 3 mg/ml tetracycline. The self-contained, tetracycline-regulated retroviral vector pBPSTR1 contains both the response unit, composed of tetracycline-resistance operon regulatory elements (*tet*O) within a minimal CMV promoter, and the regulator unit, encoding the tTA protein (the tetracycline repressor fused to the transactivator protein VP16) (Paulus *et al*., 1996). Gene expression is inhibited by tetracycline, which binds the transactivator protein tTA, causing it to dissociate from the *tet*O minimal CMV promoter. Changes in protein expression were verified by immunofluorescence staining.

*Immunofluorescence staining of breast cells*

Cells were immunostained and quantified as previously described (Bianco and Montano, 2002; Bianco *et al*., 2003). Cells grown on coverslips were fixed in 4% paraformaldehyde. After blocking with 5% normal goat serum, samples were incubated with GST-Pi or GCSh antibody (1 : 100 dilution) and goat, anti-rabbit IgG Alexa 488 fluorescent secondary antibody (Molecular Probes). As a negative control, duplicate sections were immunostained with nonspecific rabbit IgG or with secondary antibody alone. Semiquantitation analysis was performed on a MacIntosh computer using Adobe Photoshop 6.0 software. The mean luminosity of 15–20 cells from each experiment was measured and averaged with background subtracted out from each field.

*Immunocytochemistry for 8-OHDG in breast cells*

8-OHdG levels were measured and quantified as previously described (Bianco *et al*., 2003). Cells grown on coverslips were fixed in methacarn (methanol/chloroform/acetic acid, 6 : 3 : 1) for 1 h at RT. Endogenous peroxidase activity in the cells was eliminated by a 30 min incubation with 3% $H_2O_2$ in methanol, and nonspecific binding sites were blocked in a 15 min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris-HCl and 150 mM NaCl, pH 7.6). The cells were then pretreated with proteinase-K (20 $\mu$g/ml in PBS, pH 7.4 for 15 min at RT; Boehringer Mannheim, Indianapolis, IN, USA). To detect oxidized nucleosides, we used the anti-8-oxo-dG monoclonal antibody 1F7 (1 : 100; Trevigen, Gaithersburg, MD, USA). As a negative control, cells were incubated without the primary antibody. Immunostaining was developed by the peroxidase–antiperoxidase procedure.

Immunoreactivity was evaluated by measuring optical density (OD). The OD was assessed using a Carl Zeiss Axiocam digital camera with a KS300 Imaging System quantitation program. OD of manually outlined cells were measured. Five cells in three adjacent fields were measured and the background OD was subtracted from each. Each experiment was performed two or more times and results were measured under the same optical and light conditions. Statistical analysis was performed using the Students *t*-test.

*Statistical analysis*

Data were evaluated by analysis of variance and tested for statistical significance using the Student's *t*-test.

**Abbreviations**

ER$\beta$, estrogen receptor $\beta$; ER$\alpha$, estrogen receptor $\alpha$; E2, 17$\beta$-estradiol; TOT, *trans*-hydroxytamoxifen; QR, quinone reductase; GST-Pi, glutathione *S*-transferases Pi; GCSh, gamma-glutamylcysteine synthetase heavy subunit; ARE/EpRE, antioxidant/electrophile response element ; 8-OHdG, 8-hydroxydeoxyguanine.

**Acknowledgements**

We thank Dr Steven A Reeves (Massachusetts General Hospital) for the pBPSTR1 retroviral vector, Dr Henry Forman (University of Alabama) for the GCSh antibody, and Dr R Timothy Mulchay (University of Wisconsin) for the GCSh-pGL3 reporter.

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